Cell Host & Microbe
Article

Natural Killer Cells Are Required for Extramedullary Hematopoiesis following Murine Cytomegalovirus Infection

Stefan Jordan,[1,*] Zsolt Ruzsics,[1] Maja Mitrović,[2] Thomas Baranek,[3,4,5,6] Jurica Arapović,[2,7] Astrid Krmpotić,[2] Eric Vivier,[3,4,5] Marc Dalod,[3,4,5] Stipan Jonjić,[2] Lars Dölken,[1,8] and Ulrich H. Koszinowski[1]
[1]Max von Pettenkofer-Institute, Ludwig-Maximilians-Universität, 80336 Munich, Germany
[2]Department for Histology and Embryology, School of Medicine, University of Rijeka, 51000 Rijeka, Croatia
[3]Centre d'Immunologie de Marseille-Luminy, Université de la Méditerranée, UNIV UM2, 13288 Marseille, France
[4]INSERM, UMR1104, 13288 Marseille, France
[5]CNRS, UMR7282, 13288 Marseille, France
[6]Present address: Université Francois Rabelais, INSERM, Centre d'Etude des Pathologies Respiratoires, UMR1100/EA6305, 37200 Tours, France
[7]Present address: Faculty of Medicine, University of Mostar, 88000 Mostar, Bosnia and Herzegovina
[8]Present address: University of Cambridge, Department of Medicine, Addenbrooke's Hospital, Cambridge CB2 0QQ, UK
*Correspondence: jordan@mvp.uni-muenchen.de
http://dx.doi.org/10.1016/j.chom.2013.04.007

SUMMARY

The immune response against a variety of pathogens can lead to activation of blood formation at ectopic sites, a process termed extramedullary hematopoiesis (EMH). The underlying mechanisms of EMH have been enigmatic. Investigating splenic EMH in mice infected with murine cytomegalovirus (MCMV), we find that, while cells of the adaptive immune system were dispensable for EMH, natural killer (NK) cells were essential. EMH required recognition of infected cells via activating NK cell receptors Ly49H or NKG2D, and correspondingly, viral interference with NK cell recognition abolished EMH. Surprisingly, development of EMH was not induced by NK cell-derived cytokines but was dependent on perforin-mediated cytotoxicity in order to control virus spread. Spreading virus reduced the numbers of F4/80$^+$ macrophages that were crucial for inflammatory EMH. Hence, whereas MCMV suppresses inflammation-induced EMH, NK cells confine virus spread, thereby protecting extramedullary hematopoietic niches and facilitating EMH.

INTRODUCTION

Hematopoiesis is fundamental for both innate and adaptive immunity as it constantly generates the cellular constituents of the immune system. In the developing embryo, hematopoiesis first occurs in the yolk sac and later mainly in liver and spleen. In adults, the bone marrow is the main blood forming tissue (medullary hematopoiesis). Interestingly, the immune response against various bacterial (MacNamara et al., 2009; Piseddu et al., 2011), parasitic (Cotterell et al., 2000; Giordanengo et al., 2002; Villeval et al., 1990), and both acute and chronic viral pathogens (Costantini et al., 2009; Lucia and Booss, 1981) can reactivate blood formation at sites of fetal hematopoiesis—a process termed extramedullary hematopoiesis (EMH). In most cases liver and spleen resume their hematopoietic function, resulting in a substantial increase in size of these organs (hepatosplenomegaly). In addition, EMH can also be observed in lymph nodes, thymus, renal capsule, dura, and skin.

Human cytomegalovirus (HCMV), a herpesvirus of the β-subfamily, is highly prevalent worldwide and frequently causes severe disease in individuals with an immature or compromised immune system (Mocarski et al., 2007). In newborns, congenital HCMV infection can lead to so-called "blueberry muffin" babies due to hemorrhagic-purpuric looking skin lesions associated with EMH and hepatosplenomegaly (Gaffin and Gallagher, 2007). In adults, acute HCMV infection occasionally induces mononucleosis-like symptoms and splenomegaly with the risk of rupture of the splenic capsule and severe hemorrhage (Alliot et al., 2001; Duarte et al., 2003).

Murine cytomegalovirus (MCMV) resembles its human counterpart in many regards (Krmpotic et al., 2003). As for HCMV, acute MCMV infection induces splenomegaly (Leung et al., 1991), which, in mice, is due to EMH (Lucia and Booss, 1981). The mechanism governing EMH during inflammation or infection, however, is poorly understood. Here, we report on the surprising observation that natural killer (NK) cells are crucial for the development of EMH during MCMV infection.

NK cells play a pivotal role in the first line of defense against CMV by recognizing and killing infected cells as well as secreting antiviral cytokines including interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) (Vivier et al., 2011). NK cells express a plethora of receptors that stimulate (activating receptors) or dampen (inhibitory receptors) their activity (Vivier et al., 2011). Activating receptors have either cellular partners, as NK group 2D (NKG2D) that binds to several stress-induced ligands, or directly recognize virus gene products (Vivier et al., 2011). The activating NK cell receptor Ly49H, for instance, which is expressed in C57BL/6 mice, recognizes the MCMV-encoded m157 glycoprotein on the surface of infected cells mediating

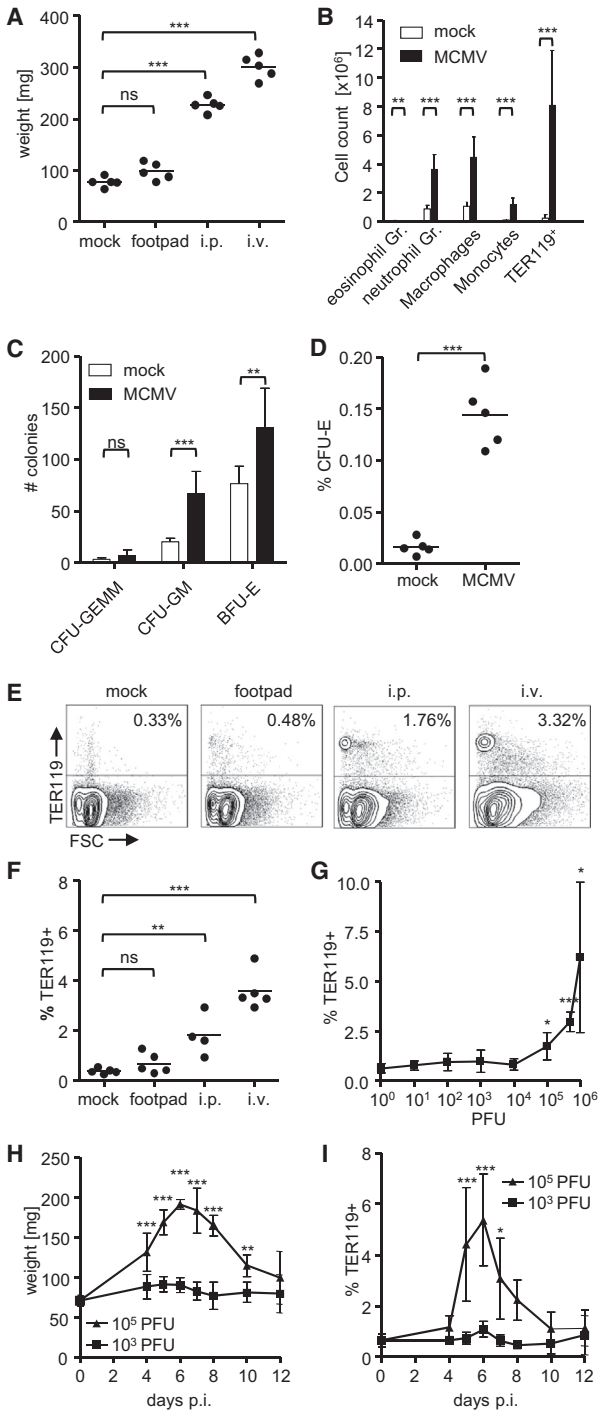

Figure 1. MCMV Infection Induces Extramedullary Hematopoiesis

C57BL/6 mice were either mock-infected or infected with MCMV injected into the footpad, intraperitoneally (i.p.), or intravenously (i.v.).

(A) Splenic weight at day 7 postinfection (p.i.) with 10^6 pfu. Each dot represents an individual animal. Horizontal bar represents mean value.

(B) Total cell numbers of eosinophil and neutrophil granulocytes (Gr.), macrophages, monocytes, and TER119$^+$ cells after i.v. infection with 10^6 pfu at day 6 (n = 5 animals; mean + SD).

(C) Mice were i.v. infected with 5×10^5 pfu MCMV. At day 6 p.i., 6×10^5 splenocytes were plated for colony-formings assays. Number of colonies is shown (n = 5 animals; mean + SD).

(D) Percentage of CFU-E at day 6 after i.v. injection with 5×10^5 pfu. Each dot represents an individual animal. Horizontal bar represents mean value.

(E and F) Analysis of TER119 expression on splenocytes at day 7 after infection with 10^6 pfu MCMV. (E) Dot plot of the animal representing the median of n = 5 animals is shown. (F) Percentage of TER119$^+$ splenocytes. Each dot represents an individual animal. Horizontal bar represents mean value.

(G) Mice were i.v. infected with the indicated doses of MCMV. Percentage of TER119$^+$ splenocytes at day 7 p.i. is shown (n = 4 animals; mean ± SD).

(H and I) Mice were i.v. infected with the indicated doses of MCMV. Kinetics of splenic weight (H) and percentage of TER119$^+$ cells (I) is shown (n = 5 animals; mean ± SD). Asterisks indicate significant values as calculated by one-way ANOVA with Dunnett test (A and F–I) or unpaired, two-tailed Student's t test (B–D): *p < 0.05; **p < 0.03; ***p < 0.001; ns, not significant.

robust activation of Ly49H$^+$ NK cells and efficient virus control (Arase et al., 2002; Bubić et al., 2004; Scalzo and Yokoyama, 2008; Smith et al., 2002).

In this study, we reveal a central role of Ly49H$^+$ NK cells in preventing MCMV-induced suppression of inflammatory EMH by restriction of virus spread.

RESULTS

Infection with MCMV Induces Extramedullary Hematopoiesis in the Spleen

MCMV causes splenomegaly and the expansion of hematopoietic islands in the enlarged spleen of acutely infected mice (Loh and Hudson, 1981; Lucia and Booss, 1981). To characterize MCMV-induced splenomegaly, we first tested different routes of infection. We applied MCMV to C57BL/6 mice into the footpad, intraperitoneally and intravenously, and weighed the spleens at 7 days postinfection (p.i.) (Figure 1A). Infection via the footpad did not result in a significant increase in splenic weight. In contrast, a significant increase in splenic weight was observed following both intraperitoneal and intravenous infection.

In order to investigate the contribution of the hematopoietic system in the observed splenomegaly, we performed flow cytometry. We quantified the total cell numbers of myeloid lineages, i.e., eosinophil (Gr-1$^-$CD115$^-$F4/80$^+$SSC^{hi}) and neutrophil (Gr-1$^+$CD115$^-$) granulocytes, macrophages (Gr-1$^-$CD115$^-$F4/80$^+$SSC^{lo}) and monocytes (Gr-1$^-$CD115$^+$) (Chow et al., 2011), as well as of the erythroid lineage (TER119$^+$) (Kina et al., 2000) in the spleen of mice after intravenous infection with MCMV. The cell numbers of all these lineages increased considerably with a remarkable dominance of the erythroid lineage (Figure 1B).

In order to confirm that the observed increase in myeloid and erythroid cell numbers after infection was indeed due to EMH, we performed colony-forming assays for hematopoietic progenitor cells from spleen homogenates. MCMV infection significantly increased the numbers of myeloid progenitors CFU-GM and erythroid progenitors BFU-E (Figure 1C). Of note, the dominance of the erythroid lineage in splenic EMH already became manifest at the level of BFU-E. BFU-E-derived CFU-E are direct precursors of TER119$^+$ erythroid cells and are accessible by flow cytometry (lin$^-$CD23$^-$CD41$^-$Sca-1$^-$c-kit$^+$CD105$^+$CD150$^-$) (Pronk et al., 2007). As expected, CFU-E were increased after MCMV infection (Figure 1D). Thus, EMH can be observed at all developmental stages from early BFU-E- to late TER119-expressing cells.

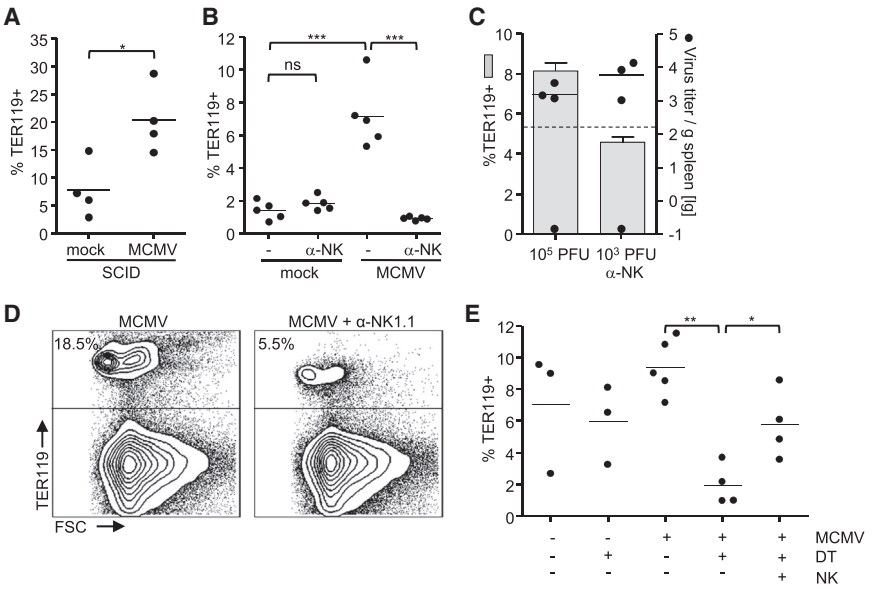

Figure 2. NK Cells Are Required for Extramedullary Hematopoiesis

(A) SCID mice were infected with MCMV. Percentage of TER119$^+$ splenocytes is shown. Each dot represents an individual animal. Horizontal bar represents mean value.

(B) C57BL/6 mice, either undepleted or NK cell-depleted using anti-asialo GM1 antibody (α-NK), were infected with MCMV. Percentage of TER119$^+$ splenocytes is shown. Each dot represents an individual animal. Horizontal bar represents mean value.

(C) C57BL/6 mice, either left undepleted or NK cell-depleted, were infected with 10^5 pfu and 10^3 pfu MCMV, respectively. Virus titer at day 3 p.i. (dots) and percentage of TER119$^+$ splenocytes at day 6 p.i. (bars) is shown. Dashed line, detection limit. Dots represent individual animals, bars represent n = 5 animals (mean + SD).

(D) C57BL/6 mice, either left undepleted or NK cell-depleted using anti-NK1.1 antibody (α-NK1.1), were infected with 2 × 10^5 pfu MCMV. TER119 expression on splenocytes at day 7 p.i. is shown.

(E) NKp46-DTR bone marrow chimeras, either left undepleted or NK cell-depleted using diphtheria toxin (DT), were either mock-infected or infected with MCMV. One group of infected NK cell-depleted mice received an adoptive transfer of wild-type NK cells (NK). Percentage of TER119$^+$ splenocytes is shown. Each dot represents an individual animal. Horizontal bar represents mean value. Asterisks indicate significant values as calculated by unpaired, two-tailed Student's t test (A and E) or one-way ANOVA with Bonferroni test (B). *$p < 0.05$; **$p < 0.03$, ***$p < 0.001$; ns, not significant.

We thus quantified the proportion of TER119$^+$ cells in the spleens of mice infected with MCMV via the footpad, intraperitoneally and intravenously (Figures 1E and 1F). As observed for spleen size, intravenous and intraperitoneal infection, but not footpad infection, resulted in expansion of the TER119$^+$ compartment in the spleen. Notably, the proportion of TER119$^+$ splenocytes nicely correlated with the splenic weight for the respective infection routes (Figure 1A), indicating that EMH substantially contributes to MCMV-induced splenomegaly. In the following, we therefore quantified the extent of EMH based on the expansion of TER119$^+$ splenocytes.

To determine the dose of infection required for the induction of EMH, we performed intravenous infections with 10^0 to 10^6 plaque-forming units (pfu) of MCMV. A significant increase in the proportion of TER119$^+$ cells was observed following infection with $\geq 10^5$ pfu (Figure 1G). Finally, we followed the splenic weight and the proportion of TER119$^+$ cells over time after low-dose (10^3 pfu) and high-dose (10^5 pfu) infection. After low-dose infection, no significant changes in splenic weight could be detected (Figure 1H). After high-dose infection, a rapid gain of weight with a peak around day 6 was seen that contracted after 10 days (Figure 1I). Thereby, the expansion of TER119$^+$ cells followed the same kinetics as the splenic weight. In summary, MCMV infection induced EMH in C57BL/6 mice in a route-, dose-, and time-dependent manner.

NK Cells Are Essential for Extramedullary Hematopoiesis upon MCMV Infection

The kinetics of EMH after MCMV infection parallels the expansion and contraction of T cells (Figure 1I) (Mohr et al., 2010). In addition, T cells and also B cells produce many cytokines with hematopoietic activity. Therefore, we tested whether T or B cells are required for the induction of EMH. Upon infection of severe combined immunodeficiency (SCID) mice that lack both T and B cells, a robust increase of TER119$^+$ splenocytes was observed (Figure 2A) indicating that cells of the adaptive immune response are dispensable for the induction of EMH following MCMV infection.

In the acute phase of an MCMV infection, innate immunity, in particular the NK cell response, is important for the containment of virus replication (Vivier et al., 2011). To examine whether NK cells contribute to the regulation of EMH, we depleted these cells using anti-asialo GM1 antibody. To our surprise, depletion of NK cells completely abolished the expansion of TER119$^+$ cells after MCMV infection (Figure 2B). This was particularly surprising because NK cell depletion in C57BL/6 mice results in a 100-fold increase in virus load. Therefore, we would have rather predicted an increase in the extent of EMH. To examine the effect of NK cell deficiency on EMH in presence of a comparable virus load of the spleen, we infected untreated mice and NK cell-depleted mice with 10^5 and 10^3 pfu MCMV, respectively (Figure 2C). Despite comparable virus loads at the peak of virus replication in the spleen (day 3 p.i.), EMH was reduced in absence of NK cells (day 6 p.i.). Therefore, the loss of EMH upon NK cell depletion was not due to an overwhelming infection. To exclude off-target effects of the anti-asialo GM1 antibody, we repeated NK cell depletion using an anti-NK1.1 antibody. As observed before, the anti-NK1.1 antibody-mediated NK cell depletion diminished the proportion of TER119$^+$ cells substantially during infection (Figure 2D). Finally, we used a genetic model for depletion of NK cells. We generated bone marrow chimeras using a graft from NKp46-DTR mice (Walzer et al., 2007). After bone marrow reconstitution, NK cells in these mice express the human diphtheria toxin receptor (DTR) and can be selectively depleted by

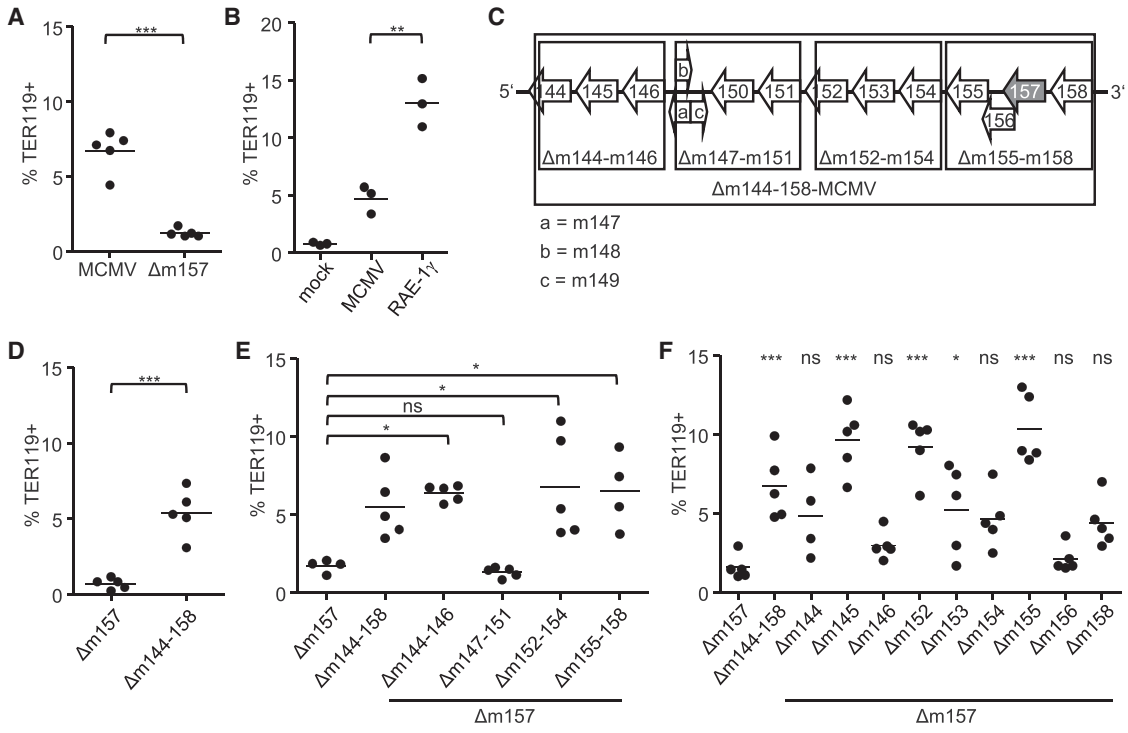

Figure 3. Ligation of Activating NK Cell Receptors Is Required for Extramedullary Hematopoiesis

(A–F) C57BL/6 mice were infected with either MCMV or the indicated MCMV mutants. Percentage of TER119$^+$ splenocytes at day 6 p.i. is shown. Each dot represents an individual animal. Horizontal bar represents mean value. Asterisks indicate significant values as calculated by unpaired, two-tailed Student's t test (A, B, D) or one-way ANOVA with Dunnett test (E, F; all groups compared to Δm157-MCMV). *$p < 0.05$; **$p < 0.03$; ***$p < 0.001$; ns, not significant. (C) Genomic organization of the locus comprising MCMV genes *m144–m158*. Arrows indicate ORFs, rectangles sequence deletions of the indicated mutants. Gene *m157* is deleted in all mutants.

injection of diphtheria toxin (DT). Of note, higher percentages of TER119$^+$ cells were already present in mock-infected chimeras irrespective of DT treatment, most likely due to a higher background level of EMH during the reconstitution phase of the hematopoietic system following bone marrow transfer (Hodek et al., 2008) (Figure 2E). Nevertheless, NK cell depletion by DT-injection significantly decreased the number of MCMV-induced TER119$^+$ cells in these mice. The genetic depletion model also enabled us to reconstitute mice depleted of NK cells with wild-type cells. Splenocytes enriched in NK cells were transferred from MCMV-infected wild-type mice into infected and NKp46-DTR-depleted chimeric mice. Consequently, this transfer restored TER119$^+$ cell expansion. In summary, these data reveal an unsuspected, crucial role of NK cells in the establishment of EMH following MCMV infection.

Recognition of MCMV-Infected Cells via the Activating NK Cell Receptors Ly49H or NKG2D Is Required for Extramedullary Hematopoiesis

In C57BL/6 mice, the Ly49H$^+$ NK cells directly recognize MCMV-infected cells due to the expression of the viral m157 protein on the cell surface of the infected cells (Arase et al., 2002; Smith et al., 2002). To investigate whether recognition of infected cells via Ly49H is important for the development of EMH, C57BL/6 mice were infected with m157-deletion virus (Δm157-MCMV) (Bubić et al., 2004). Interestingly, no expansion of TER119$^+$ cells was observed following Δm157-MCMV infection compared to wild-type virus infection (Figure 3A). Therefore, recognition of infected cells by NK cells via the Ly49H/m157 interaction seems to be important for the development of EMH.

NK cells can also be activated through ligation of NKG2D. To test directly the effect of NKG2D ligation on EMH, we infected mice with a virus expressing the NKG2D ligand RAE-1γ (RAE-1γ-MCMV) (Slavuljica et al., 2010). In these mice, the proportion of TER119$^+$ cells was significantly higher than after infection with wild-type virus (Figure 3B), demonstrating that NK cell recognition through both Ly49H and NKG2D had an additive effect.

MCMV encodes several proteins that interfere with NK cell recognition. We therefore asked whether viral interference with NK cell recognition would affect the expansion of TER119$^+$ cells in absence of Ly49H ligation. C57BL/6 mice were infected with a mutant virus lacking the genomic region *m144–m158* (Δm144–m158-MCMV) that contains a number of ORFs known or proposed to control NK cell function (Figure 3C). Interestingly, deletion of m144–m158 restored the expansion of TER119$^+$ cells (Figure 3D), indicating that efficient viral inhibition of NK cell recognition is responsible for failure to establish EMH in absence of Ly49H ligation.

To identify the responsible viral genes, we deleted smaller gene blocks dissecting the *m144–m158* region on the Δm157-MCMV background, generating the mutants Δm144–m146-, Δm147–m151-, Δm152–m154-, and Δm155–m158-MCMV

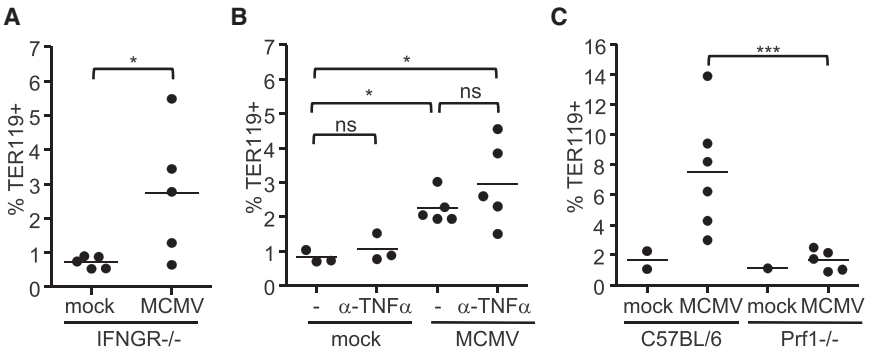

Figure 4. NK Cell-Mediated Cytotoxicity Is Required for MCMV-Induced Extramedullary Hematopoiesis

(A–C) Indicated mouse strains were infected with MCMV. (B) TNF-α was depleted in C57BL/6 mice using Etanercept (α-TNFα). Percentage of TER119$^+$ splenocytes is shown. Each dot represents an individual animal. Horizontal bar represents mean value. Asterisks indicate significant values as calculated by unpaired, two-tailed Student's t test (A and C) or one-way ANOVA with Bonferroni test (B). *$p < 0.05$; ***$p < 0.001$; ns, not significant. Data were pooled from two experiments (C).

(Figures 3C and 3E). Infection with Δm144–m146-, Δm152–m154-, and Δm155–m158-MCMV restored the expansion of TER119$^+$ cells. The viral genes *m145*, *m152*, and *m155* inhibit NK cell recognition by downregulating MULT-1, RAE-1, and H60 (Hasan et al., 2005; Krmpotic et al., 2005; Lodoen et al., 2003, 2004), respectively, all of which are ligands of the activating NK cell receptor NKG2D. Thus, each of the mutant viruses that restored the development of EMH lacked at least one ORF known to inhibit NK cell recognition. To show that deletion of these genes was indeed responsible for the development of EMH upon infection with the respective mutants, we generated single deletion mutants lacking *m145*, *m152*, or *m155* on the Δm157-MCMV background (Figures 3C and 3F). As expected, infection with all three mutants resulted in restoration of EMH. In parallel, we generated single deletion mutants of all other ORFs in *m144–m146* and *m152–m158* on the Δm157-MCMV background. None of these mutants induced expansion of TER119$^+$ cells as strongly as the Δm145-, Δm152-, and Δm155-MCMV mutants. Nevertheless, a partial rescue was observed for Δm144, Δm153, Δm154, and Δm158. Further studies are required to address whether viral genes *m144*, *m153*, *m154*, or *m158* are directly involved in the inhibition of NKG2D-mediated NK cell recognition or whether deletion of these genes simply affected expression of other genes in this genomic region known to inhibit NK cell recognition.

NK Cell-Mediated Cytotoxicity Is Essential for Extramedullary Hematopoiesis

NK cells are important producers of cytokines, e.g., IFN-γ and TNF-α, which might induce EMH. In fact, it was reported that IFN-γ can activate quiescent hematopoietic stem cells during infection (Baldridge et al., 2010). TER119$^+$ cells, however, also expanded after infection of IFNGR$^{-/-}$ mice, indicating that IFN-γ-signaling is dispensable for induction of EMH (Figure 4A). TNF-α has also been shown to act on hematopoietic stem cells after bone marrow transplantation (Pearl-Yafe et al., 2010). Yet, depletion of TNF-α had no effect on MCMV-induced EMH (Figure 4B). Besides cytokine production, perforin-mediated cytotoxicity is the most prominent function of NK cells. Therefore, we infected perforin-knockout mice (Prf1$^{-/-}$) lacking NK cell-mediated cytotoxicity (Kägi et al., 1994). Remarkably, expansion of TER119$^+$ cells was completely abrogated in Prf1$^{-/-}$ mice after MCMV infection (Figure 4C). Hence, it is the cytotoxic function of NK cells rather than cytokine production that is required for the development of EMH. This indicated that NK cell-mediated killing of MCMV-infected cells and thus direct control of MCMV-infected targets was required for the establishment of EMH.

Restriction of MCMV Spread by NK Cells Is Required for Extramedullary Hematopoiesis

NK cell-mediated cytotoxicity results in the elimination of infected cells and thereby control of virus replication and spread. To dissect whether virus replication in first target cells or spread to secondary targets affects the development of EMH, we infected mice with ΔM94-MCMV. This virus can fully replicate in cells but virus morphogenesis is interrupted at the stage of secondary envelopment that blocks virion export from cells and spread (Mohr et al., 2010). Interestingly, infection with ΔM94-MCMV resulted in the expansion of TER119$^+$ cells to a similar extent as seen for the wild-type virus (Figure 5). Yet, in contrast to wild-type virus infection, NK cell-depletion during infection with ΔM94-MCMV no longer abrogated EMH. In addition, Prf1$^{-/-}$ mice infected with ΔM94-MCMV showed normal expansion of TER119$^+$ cells. We conclude that NK cell-mediated cytotoxicity is indeed required to restrict MCMV spread in order to allow for EMH.

MCMV Escaping NK Cell Control Suppresses Inflammatory Extramedullary Hematopoiesis

Our previous data indicate that virus spread in absence of NK cell control might suppress EMH. To formally proof this hypothesis, we treated mice with synthetic CpG-motif containing oligodeoxynucleotide (CpG-ODN). CpG-ODN is a TLR9 agonist that activates cells of the adaptive and the innate immune system, and also strongly induces inflammatory EMH (Sparwasser et al., 1999). CpG-ODN-treated mice were infected with wild-type MCMV, UV-irradiated wild-type MCMV, spread-deficient ΔM94-MCMV, or Δm157-MCMV. Interestingly, infection with Δm157-MCMV completely abrogated CpG-ODN-induced expansion of TER119$^+$ cells (Figure 6A), demonstrating that MCMV escaping NK cell control suppresses inflammation-induced EMH. It is important to note that infection with wild-type MCMV also led to a lesser, but nevertheless significant reduction of CpG-ODN-induced expansion of TER119$^+$ cells. After infection with nonreplicating UV-irradiated wild-type MCMV or spread-deficient ΔM94-MCMV, neither additional expansion nor contraction of TER119$^+$ cells could be observed. Finally, we also infected CpG-ODN-treated Ly49H$^{-/-}$ mice with wild-type MCMV (Figure 6B). As predicted, MCMV infection in

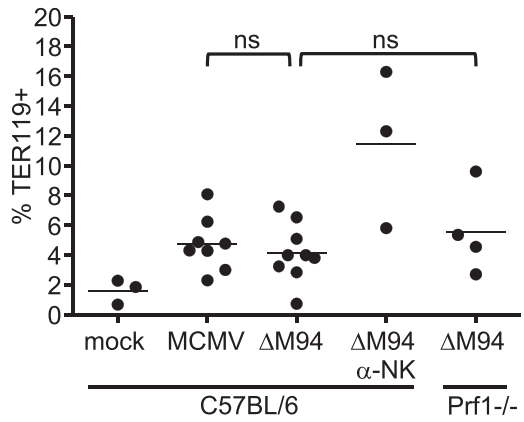

Figure 5. NK Cell-Mediated Restriction of MCMV Spread Is Required for Extramedullary Hematopoiesis

Indicated mouse strains, either left undepleted or depleted of NK cells, were infected with either MCMV or ΔM94-MCMV. Percentage of TER119$^+$ splenocytes is shown. Each dot represents an individual animal. Horizontal bar represents mean value. ns, not significant (one-way ANOVA with Bonferroni test). Data were pooled from two experiments.

Ly49H$^{-/-}$ mice led to further reduction of TER119$^+$ cells compared to C57BL/6 mice.

Our data thus indicated two consequences of an acute MCMV infection with regard to EMH. On the one hand, the inflammation associated with acute MCMV infection induced EMH in a similar fashion as treatment with TLR-agonists like CpG-ODN. On the other hand, the virus was able to suppress this inflammation-induced EMH. This suppressive effect was dependent on virus spread and was controlled by NK cell cytotoxicity.

To formally clarify the interplay between induction and suppression of EMH by MCMV, we performed double infections with MCMV and Δm157-MCMV (Figure 6C). As seen for CpG-ODN-induced EMH, Δm157-MCMV efficiently suppressed wild-type MCMV-induced expansion of TER119$^+$ cells, demonstrating that suppression of EMH is the dominant phenotype. Hence, MCMV suppresses inflammatory EMH and NK cells limit this property.

MCMV Spread Leads to the Depletion of Splenic Macrophages and Failure in LSK Cell Expansion

To gain more insight in the mechanism of viral suppression of EMH, we probed spleens of mice stimulated with CpG-ODN and infected with Δm157-MCMV for hematopoietic progenitor cells. Infection significantly reduced the proportion of erythroid CFU-E (Figure 7A) and the number of BFU-E as well as myeloid CFU-GM (Figure 7B) in the spleen as seen by flow cytometry and colony-forming assays, respectively. Notably, already the number of early multipotent CFU-GEMM was reduced in spleens from mice infected with Δm157-MCMV. Because all hematopoietic lineages descend from lineage$^-$Sca-1$^+$c-kit$^+$ (LSK) cells, we examined LSK cells in the spleen following infection with MCMV and Δm157-MCMV (Figures 7C and 7D). The proportion of LSK cells was increased after MCMV infection with a peak at day 4 p.i. followed by a rapid decrease. Interestingly, during the first 4 days of infection with Δm157-MCMV we could observe neither an increase nor a decrease of LSK cells in the spleen. Only by day 6 p.i. the percentage of LSK cells increased. The spleen, as other peripheral organs, satisfies an increased demand of hematopoietic stem cells by recruiting cells that are mobilized from the bone marrow (Schulz et al., 2009). Bone marrow pathology due to uncontrolled virus replication could lead to a diminished supply with stem cells. In fact, induction of EMH with CpG-ODN increased the number of LSK cells in the blood (Figure 7E). Infection with Δm157-MCMV, however, did not result in a reduction of circulating LSK cells, suggesting that failure of the LSK cell expansion is due to a spleen intrinsic defect.

Because virus spread was required for suppression of EMH, it was tempting to speculate that the infection of a certain cell type not infected in significant numbers during the first round of infection is responsible for suppression of EMH, e.g., the infection of hematopoietic stem cells. Furthermore, hematopoiesis requires a specialized microenvironment, the hematopoietic niche (Mercier et al., 2012). Thus, viral targeting of an integral part of the supportive microenvironment in the spleen might lead to suppression of EMH. F4/80$^+$ macrophages are key regulators of the hematopoietic niche in the bone marrow, e.g., by mediating adhesion of stem and progenitor cells (Chasis and Mohandas, 2008; Chow et al., 2011; Ehninger and Trumpp, 2011; Mercier et al., 2012). First, we wanted to know whether macrophages are indeed required for inflammatory EMH. Because macrophages could also play a role in limiting virus spread (Ebermann et al., 2012), we used CpG-ODN for sterile induction of EMH and depleted macrophages using liposome-encapsulated Clodronate (van Rooijen et al., 1989). In fact, macrophage depletion prevented the gain in splenic weight (Figure 7F) and inhibited the increase in erythroid CFU-E (Figure 7G) and TER119$^+$ cells (Figure 7H), indicating that macrophages are required for EMH and therefore a key component of the hematopoietic niche in the spleen. Macrophages are important target cells of MCMV for lytic replication (Hsu et al., 2009; Ménard et al., 2003). Hence, we examined the macrophage compartment in the spleen after infection with MCMV and Δm157-MCMV (Figure 7I). Following MCMV infection, the proportion of F4/80$^+$ macrophages remained constant and was increased only at day 6 p.i.. In contrast, after infection with Δm157-MCMV, the macrophage compartment collapsed as early as day 2 p.i. These data suggested that virus spread impacts on a constitutive element of the hematopoietic niche in the spleen, the macrophage.

DISCUSSION

EMH, during the course of acute or chronic infections, is a widely recognized phenomenon. The underlying mechanisms, however, have been enigmatic. Here, we report an unexpected role of NK cells for EMH following CMV infection.

Infection with MCMV, a β-herpesvirus, induced EMH in the spleen with a dominance of the red blood cell lineage consistent with observations made after bacterial infection (Jackson et al., 2010; MacNamara et al., 2009). The kinetics of EMH developed in parallel to the adaptive immune response, yet B and T cells were dispensable for the establishment of EMH. In contrast, we observed a striking dependence of EMH on NK cells.

In C57BL/6 mice, Ly49H$^+$ NK cells govern the early immune response against MCMV (Vivier et al., 2011) as they are able

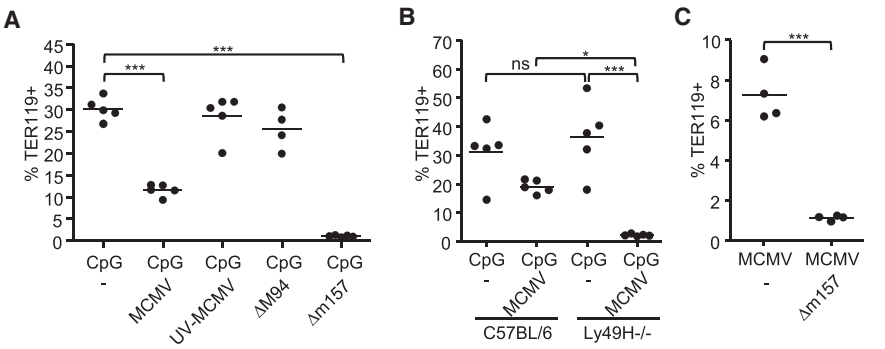

Figure 6. MCMV Escaping NK Cell Control Dominantly Suppresses CpG-ODN-Induced Extramedullary Hematopoiesis

(A) Mice were treated with CpG-ODN and either left uninfected or were infected with 5 × 10^5 pfu either untreated or UV-irradiated (UV) MCMV or the indicated MCMV mutants.

(B) Indicated mouse strains were treated with CpG-ODN and either left uninfected or were infected with 5 × 10^5 pfu MCMV.

(C) C57BL/6 mice were infected with either 10^5 pfu MCMV or 10^5 pfu MCMV + 10^5 pfu Δm157-MCMV. Percentage of TER119$^+$ splenocytes is shown. Each dot represents an individual animal. Horizontal bar represents mean value. Asterisks indicate significant values as calculated by one-way ANOVA with Bonferroni test (A and B) or unpaired, two-tailed Student's t test (C): *p < 0.05; ***p < 0.001; ns, not significant. (A) Data were pooled from two experiments (A).

to directly recognize the viral m157 protein on the surface of infected cells resulting in reduction of viral load and preservation of the microarchitecture of the splenic white pulp (Bekiaris et al., 2008; Benedict et al., 2006). Recognition of infected cells via the m157/Ly49H interaction was also critical for the development of EMH following MCMV infection. Upon infection with Δm157-MCMV that escapes recognition and control by Ly49H$^+$ NK cells, no EMH was observed. Therefore, the role of Ly49H for NK cell activation and EMH was essential in C57BL/6 mice. Other mechanisms, however, can contribute to NK cell recognition as well, such as NKG2D engagement. Our nonbiased screen of virus deletion mutants for the establishment of EMH in fact confirmed the importance of NKG2D ligation via MULT-1, RAE-1, and H60. The deletion of only one of the viral genes *m145*, *m152*, or *m155* restored EMH, even in absence of *m157*. These genes downregulate the NKG2D ligands MULT-1, RAE-1, and H60, respectively (Hasan et al., 2005; Krmpotic et al., 2005; Lodoen et al., 2003, 2004). Our data now show that all three genes—*m145*, *m152*, and *m155*—are required for MCMV to escape from NK cell recognition in vivo, and that neither of these viral products alone is sufficient for this function. Apparently, induction and suppression of EMH provides a very sensitive read-out system to study the functionality of NK cell activating ligands and their viral counter-regulators in vivo.

Cytokines produced by NK cells were proposed to have an effect on hematopoiesis (Murphy and Longo, 1996). To our great surprise, MCMV-induced EMH did not depend on cytokines but only on perforin-mediated cytotoxicity. NK cells use their cytotoxic function to confine MCMV spread by lysing infected cells. Using the spread-deficient ΔM94-MCMV mutant, we were able to confirm that containment of viral spread is indeed essential for the development of inflammation-induced EMH. Interestingly, NK cell-derived IFN-γ also restricts MCMV replication (Loh et al., 2005) but was not sufficient to substitute for perforin-mediated cytotoxicity. This might be due to organ-specific control of MCMV replication by IFN-γ and perforin in liver and spleen, respectively (Tay and Welsh, 1997). In summary, our data demonstrate a specific role of a defined NK cell effector function, namely cytolysis, to prevent suppression of EMH by MCMV.

In contrast to EMH, the effect of MCMV infection on medullary hematopoiesis has been investigated extensively (Mayer et al., 1997; Mori et al., 1999; Mutter et al., 1988; Reddehase et al., 1992). Interestingly, in mice deficient for Natural Killer T (NKT) cells or activation of NKT cells, hematopoiesis was found to be suppressed by MCMV whereas adoptive transfer of NKT cells lifted suppression (Broxmeyer et al., 2007). In addition to production of hematopoietic growth factors (Kotsianidis et al., 2006; Leite-de-Moraes et al., 2002), NKT cells can help NK cells to limit MCMV infection, particularly at later time points of infection (van Dommelen et al., 2003). Yet, it remains to be elucidated whether cytokine production or also cell lysis is the NKT cell effector function that preserves hematopoiesis upon MCMV infection.

Bone marrow failure due to HCMV infection is a dreaded complication after allogeneic bone marrow transplantation and HCMV has long been associated with suppression of hematopoiesis (Randolph-Habecker et al., 2002). In vitro, this was attributed either to the direct infection of early hematopoietic progenitors (Maciejewski and St Jeor, 1999) or of the supportive microenvironment (Smirnov et al., 2007). Lytic infection of hematopoietic stem cells or of supportive stromal cells by MCMV might also explain the suppression of EMH in absence of NK cell control. Bone marrow resident hematopoietic stem and progenitor cells depend on specific microanatomical niches for proliferation and differentiation (Ehninger and Trumpp, 2011). F4/80$^+$ macrophages are a critical element of those niches in the bone marrow. They mediate the adhesion of stem and progenitor cells and engulf the expulsed nuclei of erythroid precursor cells (Chasis and Mohandas, 2008). In line with two recent publications (Chow et al., 2013; Ramos et al., 2013), we found that macrophages were also required for the development of splenic EMH. Interestingly, the number of F4/80$^+$ macrophages was reduced in the spleen of infected mice in absence of NK cell control. Future studies will elude on the mechanism of macrophage depletion and its role for suppression of EMH.

In summary, we propose the following model: MCMV infection causes inflammation that leads to the induction of EMH, presumably mediated by proinflammatory cytokines with hematopoietic activity. Virus spread to certain secondary target cells suppresses this inflammation-induced EMH. This could be due to lytic infection of hematopoietic stem and progenitor cells, or destruction of the microanatomical niche by direct or indirect depletion of constitutive elements, e.g., macrophages. Efficient containment of virus spread by NK cell-mediated cytotoxicity rescues inflammation-induced EMH.

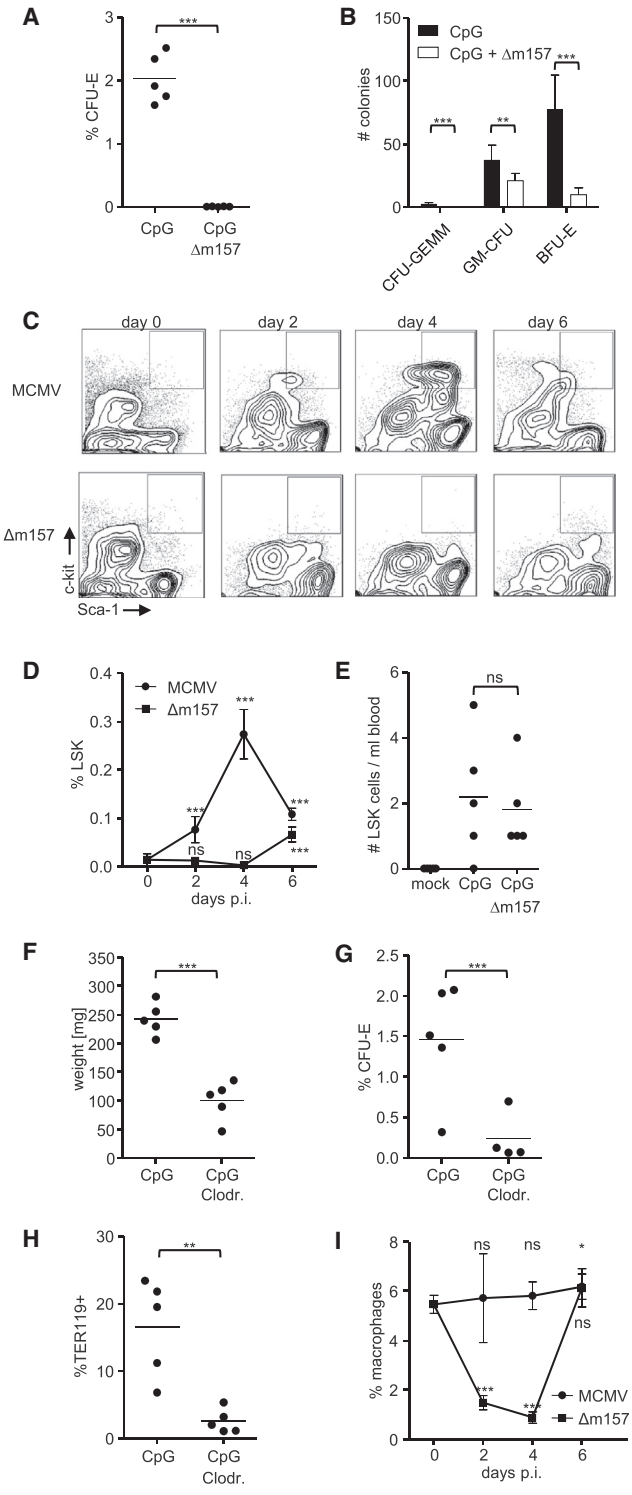

Figure 7. Absence of LSK Cell Expansion and Depletion of Splenic Macrophages during MCMV-Induced Suppression of Extramedullary Hematopoiesis

(A and B) C57BL/6 mice were treated with CpG-ODN and either left uninfected or were infected with Δm157-MCMV. (A) Percentage of CFU-E is shown. Each dot represents an individual animal. Horizontal bar represents mean value. (B) Number of colonies generated from 1 × 10^5 splenocytes (n = 5 animals; mean + SD).

(C and D) C57BL/6 mice were infected with either MCMV or Δm157-MCMV. Analysis of LSK cells at the indicated time points. (C) Dot plot of the animal closest to the mean of n = 4 animals is shown. (D) Percentage of LSK cells (n = 4 animals; mean ±− SD). Each value compared to the value at t = 0.

(E) Mice were stimulated with CpG-ODN and either left uninfected or were infected with Δm157-MCMV. Total number of LSK cells/ml in blood is shown. Each dot represents an individual animal. Horizontal bar represents mean value.

(F–H) Mice were stimulated with CpG-ODN and either left undepleted or were depleted of macrophages using liposome-encapsulated clodronate (Clodr.). (F) Spleen weight, (G) percentage of CFU-E and (H) percentage of TER119+ cells is shown. Each dot represents an individual animal. Horizontal bar represents mean value.

(I) C57BL/6 mice were infected with either MCMV or Δm157-MCMV. Percentage of macrophages in the spleen at the indicated time points (n = 4–6 animals; mean ± SD). Each value compared to the value at t = 0. Asterisks indicate significant values as calculated by unpaired, two-tailed Student's t test: *p < 0.05; **p < 0.03; ***p < 0.001; ns, not significant.

EXPERIMENTAL PROCEDURES

Mice

Female C57BL/6 mice were purchased from Elevage Janvier at the age of 7 weeks and housed at the animal facility of the Max von Pettenkofer-Institute for at least 1 week before the experiments. IFNGR$^{-/-}$ mice (Huang et al., 1993) were bred at the Max von Pettenkofer-Institute. SCID, Prf1$^{-/-}$ (Kägi et al., 1994) and Ly49H$^{-/-}$ (Fodil-Cornu et al., 2008) mice were bred at the Department for Histology and Embryology (University of Rijeka). For the generation of NKp46-DTR → C57BL/6 bone marrow chimeras recipient mice were lethally γ-irradiated (16 Gy) and reconstituted with 5 × 10^6 cells depleted of red blood cells from donors with >85% of their NK cells being hDTR+ at the Centre d'Immunologie de Marseille-Luminy (Université de la Méditerranée). BMC were used 10 weeks after reconstitution for experiments. IFNGR$^{-/-}$ mice were maintained on the 129 background, other transgenic and knockout mice on the C57BL/6 background and kept under specified-pathogen-free conditions. Experiments were performed with sex- and age-matched groups. Animal experiments were approved by the State of Bavaria or by the Ethics Committee of the respective universities.

Viruses

MCMV and MCMV mutants were derived from the molecular clone pSM3fr (Messerle et al., 1997). The construction of Δm157-MCMV, ΔM94-MCMV, and RAE–1γ-MCMV mutants was described elsewhere (Bubić et al., 2004; Mohr et al., 2010; Slavuljica et al., 2010). Further virus mutants on Δm157-MCMV background used in this study were constructed as follows: a kanamycin or ampicillin cassette was amplified by PCR from vector pACYC177 or vector pgalK-Kn (EMBL accession numbers X06402 and FR832405), respectively, with primers containing 50 bp homology to the regions flanking the sequence to be deleted (for sequences, see Table S1 available online). The deletion preserved presumed promoter sequences of neighboring genes. The PCR product was used for BAC-recombination as described before (Warming et al., 2005) with modification (Lemnitzer et al., 2013). Mouse embryo fibroblasts (MEFs) were transfected with the respective BACs to reconstitute the viruses. MCMV and MCMV mutants were propagated on M2-10B4 or NT/M94-7 cells as described (Cicin-Sain et al., 2005; Mohr et al., 2010). Virus quantification was done by plaque assay on MEFs or NT/M94-7 cells. UV-irradiation of viruses was performed as described elsewhere (Mohr et al., 2010).

Infection, In Vivo Treatments of Mice, and Organ Harvest

If not indicated otherwise, infections with MCMV and MCMV mutants were performed with 10^5 pfu in a total volume of 300 μl PBS into the tail vein. Injection of CpG-ODN 1826 (10 nmol; Tib Molbiol) was performed after anesthesia with Isoflurane into the right hind footpad in a volume of 25 μl PBS. NK cells were depleted in C57BL/6 mice by i.p. application of 25 μg anti-asialo GM1 antibody (Wako Chemicals) or 300 μg PK136 antibody, or 1 μg DT (Calbiochem) in NKp46-DTR bone marrow chimeras 1 day before and 3 days after

infection. TNF-α was depleted using Enbrel (Etanercept, Wyeth) (Plater-Zyberk et al., 2009). Macrophages were depleted by i.p. application of 250 μl liposome-encapsulated Clodronate (http://ClodronateLiposomes.com) at days −2, 0, and 3 (van Rooijen et al., 1989). Spleens were analyzed at day 6 p.i. if not indicated otherwise. Infectious virus load in spleens was determined as described previously (Cicin-Sain et al., 2005). Colony-forming assays were performed according to the manufacturer's instructions (Stem Cell Technologies).

Adoptive Transfer of Cells

For adoptive transfer of NK cells, spleens from mice that were infected for 48 hr were harvested and homogenized. Splenocytes were enriched in NK cells up to 80% using an NK cell isolation kit (Miltenyi) according to the manufacturer's instructions. Cells (10^6) were transferred at 48 hr p.i.

Flow Cytometry

Antibodies specific for the markers B220 (RA3-6B2), CD3 (145-2C11), CD5 (53-7.3), CD11b (M1/70), CD23 (B3B4), CD41 (eBioMWReg30), CD105 (MJ7/18), CD115 (AFS98), c-kit (2D8), CD150 (9D1), F4/80 (BM8), Gr-1 (RB6-8C5), NK1.1 (PK136), Sca-1 (D7), and TER119 (TER-119) were purchased from eBiosciences. Splenocytes were stained after lysis of erythrocytes with ACK-lysing buffer (155 mM ammonium chloride, 10 mM potassium bicarbonate, 0.1 mM EDTA, adjusted to pH7.2–7.4 using hydrochloric acid). Flow cytometry was performed on a FACSCalibur or a FACSCanto II (Becton Dickinson) using the BD CellQuest Pro or the BD FACSDiva Software and data analysis was carried out using the FlowJo Software (Tree Star).

Statistical Analysis

Statistical analyses were done using Prism 5 (GraphPad Software). For all experiments, the mean values were calculated. To test for significance either an unpaired, two-tailed Student's t test or ANOVA with Bonferroni or Dunnett test were used according to the data set.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one table and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2013.04.007.

ACKNOWLEDGMENTS

We thank Natalie Röder, Madlen Pogoda, Olaf Alberto von Mankowski, Barbara Meier, and the Animal Facility of the Max von Pettenkofer-Institute for excellent technical assistance and Torsten Sacher, Christian Mohr, and Wolfgang Kastenmüller for helpful discussions. This work was supported by the Studienstiftung des deutschen Volkes (S. Jordan), NGFN grant 01GS0801 (L.D., U.H.K.), the Bayerische Forschungsstiftung via Forprotect TH-1 and TH-2 (S. Jordan, Z.R., U.H.K.), the Croatian Science Foundation (grant 04/16, S. Jonjic), the Croatian ministry of science, education and sports (grant 0621261-1268, A.K.) and the French National Research Agency (grant ANR-08-MIEN-008-02, project EMICIF, M.D.).

Received: November 27, 2012
Revised: March 18, 2013
Accepted: April 12, 2013
Published: May 15, 2013

REFERENCES

Alliot, C., Beets, C., Besson, M., and Derolland, P. (2001). Spontaneous splenic rupture associated with CMV infection: report of a case and review. Scand. J. Infect. Dis. *33*, 875–877.

Arase, H., Mocarski, E.S., Campbell, A.E., Hill, A.B., and Lanier, L.L. (2002). Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. Science *296*, 1323–1326.

Baldridge, M.T., King, K.Y., Boles, N.C., Weksberg, D.C., and Goodell, M.A. (2010). Quiescent haematopoietic stem cells are activated by IFN-gamma in response to chronic infection. Nature *465*, 793–797.

Bekiaris, V., Timoshenko, O., Hou, T.Z., Toellner, K., Shakib, S., Gaspal, F., McConnell, F.M., Parnell, S.M., Withers, D., Buckley, C.D., et al. (2008). Ly49H+ NK cells migrate to and protect splenic white pulp stroma from murine cytomegalovirus infection. J. Immunol. *180*, 6768–6776.

Benedict, C.A., De Trez, C., Schneider, K., Ha, S., Patterson, G., and Ware, C.F. (2006). Specific remodeling of splenic architecture by cytomegalovirus. PLoS Pathog. *2*, e16.

Broxmeyer, H.E., Dent, A., Cooper, S., Hangoc, G., Wang, Z.Y., Du, W., Gervay-Haque, J., Sriram, V., Renukaradhya, G.J., and Brutkiewicz, R.R. (2007). A role for natural killer T cells and CD1d molecules in counteracting suppression of hematopoiesis in mice induced by infection with murine cytomegalovirus. Exp. Hematol. *35*(4, Suppl 1), 87–93.

Bubić, I., Wagner, M., Krmpotić, A., Saulig, T., Kim, S., Yokoyama, W.M., Jonjić, S., and Koszinowski, U.H. (2004). Gain of virulence caused by loss of a gene in murine cytomegalovirus. J. Virol. *78*, 7536–7544.

Chasis, J.A., and Mohandas, N. (2008). Erythroblastic islands: niches for erythropoiesis. Blood *112*, 470–478.

Chow, A., Lucas, D., Hidalgo, A., Méndez-Ferrer, S., Hashimoto, D., Scheiermann, C., Battista, M., Leboeuf, M., Prophete, C., van Rooijen, N., et al. (2011). Bone marrow CD169+ macrophages promote the retention of hematopoietic stem and progenitor cells in the mesenchymal stem cell niche. J. Exp. Med. *208*, 261–271.

Chow, A., Huggins, M., Ahmed, J., Hashimoto, D., Lucas, D., Kunisaki, Y., Pinho, S., Leboeuf, M., Noizat, C., van Rooijen, N., et al. (2013). $CD169^+$ macrophages provide a niche promoting erythropoiesis under homeostasis and stress. Nat Med. *19*, 429–436.

Cicin-Sain, L., Podlech, J., Messerle, M., Reddehase, M.J., and Koszinowski, U.H. (2005). Frequent coinfection of cells explains functional in vivo complementation between cytomegalovirus variants in the multiply infected host. J. Virol. *79*, 9492–9502.

Costantini, A., Giuliodoro, S., Butini, L., Silvestri, G., Leoni, P., and Montroni, M. (2009). Abnormalities of erythropoiesis during HIV-1 disease: a longitudinal analysis. J. Acquir. Immune Defic. Syndr. *52*, 70–74.

Cotterell, S.E., Engwerda, C.R., and Kaye, P.M. (2000). Enhanced hematopoietic activity accompanies parasite expansion in the spleen and bone marrow of mice infected with Leishmania donovani. Infect. Immun. *68*, 1840–1848.

Duarte, P.J., Echavarría, M., Paparatto, A., and Cacchione, R. (2003). [Spontaneous spleen rupture associated to active cytomegalovirus infection]. Medicina (B. Aires) *63*, 46–48.

Ebermann, L., Ruzsics, Z., Guzmán, C.A., van Rooijen, N., Casalegno-Garduño, R., Koszinowski, U., and Čičin-Šain, L. (2012). Block of death-receptor apoptosis protects mouse cytomegalovirus from macrophages and is a determinant of virulence in immunodeficient hosts. PLoS Pathog. *8*, e1003062.

Ehninger, A., and Trumpp, A. (2011). The bone marrow stem cell niche grows up: mesenchymal stem cells and macrophages move in. J. Exp. Med. *208*, 421–428.

Fodil-Cornu, N., Lee, S.H., Belanger, S., Makrigiannis, A.P., Biron, C.A., Buller, R.M., and Vidal, S.M. (2008). Ly49h-deficient C57BL/6 mice: a new mouse cytomegalovirus-susceptible model remains resistant to unrelated pathogens controlled by the NK gene complex. J. Immunol. *181*, 6394–6405.

Gaffin, J.M., and Gallagher, P.G. (2007). Picture of the month. Blueberry muffin baby (extramedullary hematopoiesis) due to congenital cytomegalovirus infection. Arch. Pediatr. Adolesc. Med. *161*, 1102–1103.

Giordanengo, L., Guiñazú, N., Stempin, C., Fretes, R., Cerbán, F., and Gea, S. (2002). Cruzipain, a major Trypanosoma cruzi antigen, conditions the host immune response in favor of parasite. Eur. J. Immunol. *32*, 1003–1011.

Hasan, M., Krmpotic, A., Ruzsics, Z., Bubic, I., Lenac, T., Halenius, A., Loewendorf, A., Messerle, M., Hengel, H., Jonjic, S., and Koszinowski, U.H. (2005). Selective down-regulation of the NKG2D ligand H60 by mouse cytomegalovirus m155 glycoprotein. J. Virol. *79*, 2920–2930.

Hodek, M., Vávrová, J., Sinkorová, Z., Mokrý, J., and Filip, S. (2008). Hematopoietic recovery after transplantation CD117+B220- (LACZ*) bone marrow cells in lethally irradiated mice. Acta Med. (Hradec Kralove) *51*, 37–41.

Hsu, K.M., Pratt, J.R., Akers, W.J., Achilefu, S.I., and Yokoyama, W.M. (2009). Murine cytomegalovirus displays selective infection of cells within hours after systemic administration. J. Gen. Virol. *90*, 33–43.

Huang, S., Hendriks, W., Althage, A., Hemmi, S., Bluethmann, H., Kamijo, R., Vilcek, J., Zinkernagel, R.M., and Aguet, M. (1993). Immune response in mice that lack the interferon-gamma receptor. Science *259*, 1742–1745.

Jackson, A., Nanton, M.R., O'Donnell, H., Akue, A.D., and McSorley, S.J. (2010). Innate immune activation during Salmonella infection initiates extramedullary erythropoiesis and splenomegaly. J. Immunol. *185*, 6198–6204.

Kägi, D., Ledermann, B., Bürki, K., Seiler, P., Odermatt, B., Olsen, K.J., Podack, E.R., Zinkernagel, R.M., and Hengartner, H. (1994). Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. Nature *369*, 31–37.

Kina, T., Ikuta, K., Takayama, E., Wada, K., Majumdar, A.S., Weissman, I.L., and Katsura, Y. (2000). The monoclonal antibody TER-119 recognizes a molecule associated with glycophorin A and specifically marks the late stages of murine erythroid lineage. Br. J. Haematol. *109*, 280–287.

Kotsianidis, I., Silk, J.D., Spanoudakis, E., Patterson, S., Almeida, A., Schmidt, R.R., Tsatalas, C., Bourikas, G., Cerundolo, V., Roberts, I.A., and Karadimitris, A. (2006). Regulation of hematopoiesis in vitro and in vivo by invariant NKT cells. Blood *107*, 3138–3144.

Krmpotic, A., Bubic, I., Polic, B., Lucin, P., and Jonjic, S. (2003). Pathogenesis of murine cytomegalovirus infection. Microbes Infect. *5*, 1263–1277.

Krmpotic, A., Hasan, M., Loewendorf, A., Saulig, T., Halenius, A., Lenac, T., Polic, B., Bubic, I., Kriegeskorte, A., Pernjak-Pugel, E., et al. (2005). NK cell activation through the NKG2D ligand MULT-1 is selectively prevented by the glycoprotein encoded by mouse cytomegalovirus gene m145. J. Exp. Med. *201*, 211–220.

Leite-de-Moraes, M.C., Lisbonne, M., Arnould, A., Machavoine, F., Herbelin, A., Dy, M., and Schneider, E. (2002). Ligand-activated natural killer T lymphocytes promptly produce IL-3 and GM-CSF in vivo: relevance to peripheral myeloid recruitment. Eur. J. Immunol. *32*, 1897–1904.

Lemnitzer, F., Raschbichler, V., Kolodziejczak, D., Israel, L., Imhof, A., Bailer, S.M., Koszinowski, U., and Ruzsics, Z. (2013). Mouse cytomegalovirus egress protein pM50 interacts with cellular endophilin-A2. Cell. Microbiol. *15*, 335–351.

Leung, W.C., Hashimoto, K., Umehara, K., and Hata, J. (1991). Murine cytomegalovirus infection model in Balb/c mice. 3. Immunoglobulin production during infection. Tokai J. Exp. Clin. Med. *16*, 11–20.

Lodoen, M., Ogasawara, K., Hamerman, J.A., Arase, H., Houchins, J.P., Mocarski, E.S., and Lanier, L.L. (2003). NKG2D-mediated natural killer cell protection against cytomegalovirus is impaired by viral gp40 modulation of retinoic acid early inducible 1 gene molecules. J. Exp. Med. *197*, 1245–1253.

Lodoen, M.B., Abenes, G., Umamoto, S., Houchins, J.P., Liu, F., and Lanier, L.L. (2004). The cytomegalovirus m155 gene product subverts natural killer cell antiviral protection by disruption of H60-NKG2D interactions. J. Exp. Med. *200*, 1075–1081.

Loh, L., and Hudson, J.B. (1981). Murine cytomegalovirus infection in the spleen and its relationship to immunosuppression. Infect. Immun. *32*, 1067–1072.

Loh, J., Chu, D.T., O'Guin, A.K., Yokoyama, W.M., and Virgin, H.W., 4th. (2005). Natural killer cells utilize both perforin and gamma interferon to regulate murine cytomegalovirus infection in the spleen and liver. J. Virol. *79*, 661–667.

Lucia, H.L., and Booss, J. (1981). Immune stimulation, inflammation, and changes in hematopoiesis. Host responses of the murine spleen to infection with cytomegalovirus. Am. J. Pathol. *104*, 90–97.

Maciejewski, J.P., and St Jeor, S.C. (1999). Human cytomegalovirus infection of human hematopoietic progenitor cells. Leuk. Lymphoma *33*, 1–13.

MacNamara, K.C., Racine, R., Chatterjee, M., Borjesson, D., and Winslow, G.M. (2009). Diminished hematopoietic activity associated with alterations in innate and adaptive immunity in a mouse model of human monocytic ehrlichiosis. Infect. Immun. *77*, 4061–4069.

Mayer, A., Podlech, J., Kurz, S., Steffens, H.P., Maiberger, S., Thalmeier, K., Angele, P., Dreher, L., and Reddehase, M.J. (1997). Bone marrow failure by cytomegalovirus is associated with an in vivo deficiency in the expression of essential stromal hemopoietin genes. J. Virol. *71*, 4589–4598.

Ménard, C., Wagner, M., Ruzsics, Z., Holak, K., Brune, W., Campbell, A.E., and Koszinowski, U.H. (2003). Role of murine cytomegalovirus US22 gene family members in replication in macrophages. J. Virol. *77*, 5557–5570.

Mercier, F.E., Ragu, C., and Scadden, D.T. (2012). The bone marrow at the crossroads of blood and immunity. Nat. Rev. Immunol. *12*, 49–60.

Messerle, M., Crnkovic, I., Hammerschmidt, W., Ziegler, H., and Koszinowski, U.H. (1997). Cloning and mutagenesis of a herpesvirus genome as an infectious bacterial artificial chromosome. Proc. Natl. Acad. Sci. USA *94*, 14759–14763.

Mocarski, E.S., Shenk, T., and Pass, R.F. (2007). Cytomegaloviruses. In Fields Virology, D.M. Knipe and P.M. Howley, eds. (Philadelphia, PA: Lippincott Williams and Wilkins), pp. 2701–2773.

Mohr, C.A., Arapovic, J., Mühlbach, H., Panzer, M., Weyn, A., Dölken, L., Krmpotic, A., Voehringer, D., Ruzsics, Z., Koszinowski, U., and Sacher, T. (2010). A spread-deficient cytomegalovirus for assessment of first-target cells in vaccination. J. Virol. *84*, 7730–7742.

Mori, T., Nakamura, M., Shimizu, K., Ikeda, Y., and Ando, K. (1999). In vivo disturbance of hematopoiesis in mice persistently infected with murine cytomegalovirus: impairment of stromal cell function. Virology *253*, 145–154.

Murphy, W.J., and Longo, D.L. (1996). NK Cells in the Regulation of Hematopoiesis. Methods *9*, 344–351.

Mutter, W., Reddehase, M.J., Busch, F.W., Bühring, H.J., and Koszinowski, U.H. (1988). Failure in generating hemopoietic stem cells is the primary cause of death from cytomegalovirus disease in the immunocompromised host. J. Exp. Med. *167*, 1645–1658.

Pearl-Yafe, M., Mizrahi, K., Stein, J., Yolcu, E.S., Kaplan, O., Shirwan, H., Yaniv, I., and Askenasy, N. (2010). Tumor necrosis factor receptors support murine hematopoietic progenitor function in the early stages of engraftment. Stem Cells *28*, 1270–1280.

Piseddu, E., Trotta, M., Tortoli, E., Avanzi, M., Tasca, S., and Solano-Gallego, L. (2011). Detection and molecular characterization of Mycobacterium celatum as a cause of splenitis in a domestic ferret (Mustela putorius furo). J. Comp. Pathol. *144*, 214–218.

Plater-Zyberk, C., Joosten, L.A., Helsen, M.M., Koenders, M.I., Baeuerle, P.A., and van den Berg, W.B. (2009). Combined blockade of granulocyte-macrophage colony stimulating factor and interleukin 17 pathways potently suppresses chronic destructive arthritis in a tumour necrosis factor alpha-independent mouse model. Ann. Rheum. Dis. *68*, 721–728.

Pronk, C.J., Rossi, D.J., Månsson, R., Attema, J.L., Norddahl, G.L., Chan, C.K., Sigvardsson, M., Weissman, I.L., and Bryder, D. (2007). Elucidation of the phenotypic, functional, and molecular topography of a myeloerythroid progenitor cell hierarchy. Cell Stem Cell *1*, 428–442.

Ramos, P., Casu, C., Gardenghi, S., Breda, L., Crielaard, B.J., Guy, E., Marongiu, M.F., Gupta, R., Levine, R.L., Abdel-Wahab, O., et al. (2013). Macrophages support pathological erythropoiesis in polycythemia vera and β-thalassemia. Nat Med. *19*, 437–445.

Randolph-Habecker, J., Iwata, M., and Torok-Storb, B. (2002). Cytomegalovirus mediated myelosuppression. J. Clin. Virol. *25*(*Suppl 2*), S51–S56.

Reddehase, M.J., Dreher-Stumpp, L., Angele, P., Balthesen, M., and Susa, M. (1992). Hematopoietic stem cell deficiency resulting from cytomegalovirus infection of bone marrow stroma. Ann. Hematol. Suppl. *64*, A125–A127.

Scalzo, A.A., and Yokoyama, W.M. (2008). Cmv1 and natural killer cell responses to murine cytomegalovirus infection. Curr. Top. Microbiol. Immunol. *321*, 101–122.

Schulz, C., Von Andrian, U.H., and Massberg, S. (2009). Trafficking of murine hematopoietic stem and progenitor cells in health and vascular disease. Microcirculation *16*, 497–507.

Slavuljica, I., Busche, A., Babić, M., Mitrović, M., Gašparović, I., Cekinović, D., Markova Car, E., Pernjak Pugel, E., Ciković, A., Lisnić, V.J., et al. (2010). Recombinant mouse cytomegalovirus expressing a ligand for the NKG2D

receptor is attenuated and has improved vaccine properties. J. Clin. Invest. *120*, 4532–4545.

Smirnov, S.V., Harbacheuski, R., Lewis-Antes, A., Zhu, H., Rameshwar, P., and Kotenko, S.V. (2007). Bone-marrow-derived mesenchymal stem cells as a target for cytomegalovirus infection: implications for hematopoiesis, self-renewal and differentiation potential. Virology *360*, 6–16.

Smith, H.R., Heusel, J.W., Mehta, I.K., Kim, S., Dorner, B.G., Naidenko, O.V., Iizuka, K., Furukawa, H., Beckman, D.L., Pingel, J.T., et al. (2002). Recognition of a virus-encoded ligand by a natural killer cell activation receptor. Proc. Natl. Acad. Sci. USA *99*, 8826–8831.

Sparwasser, T., Hültner, L., Koch, E.S., Luz, A., Lipford, G.B., and Wagner, H. (1999). Immunostimulatory CpG-oligodeoxynucleotides cause extramedullary murine hemopoiesis. J. Immunol. *162*, 2368–2374.

Tay, C.H., and Welsh, R.M. (1997). Distinct organ-dependent mechanisms for the control of murine cytomegalovirus infection by natural killer cells. J. Virol. *71*, 267–275.

van Dommelen, S.L., Tabarias, H.A., Smyth, M.J., and Degli-Esposti, M.A. (2003). Activation of natural killer (NK) T cells during murine cytomegalovirus infection enhances the antiviral response mediated by NK cells. J. Virol. *77*, 1877–1884.

van Rooijen, N., Kors, N., and Kraal, G. (1989). Macrophage subset repopulation in the spleen: differential kinetics after liposome-mediated elimination. J. Leukoc. Biol. *45*, 97–104.

Villeval, J.L., Lew, A., and Metcalf, D. (1990). Changes in hemopoietic and regulator levels in mice during fatal or nonfatal malarial infections. I. Erythropoietic populations. Exp. Parasitol. *71*, 364–374.

Vivier, E., Raulet, D.H., Moretta, A., Caligiuri, M.A., Zitvogel, L., Lanier, L.L., Yokoyama, W.M., and Ugolini, S. (2011). Innate or adaptive immunity? The example of natural killer cells. Science *331*, 44–49.

Walzer, T., Bléry, M., Chaix, J., Fuseri, N., Chasson, L., Robbins, S.H., Jaeger, S., André, P., Gauthier, L., Daniel, L., et al. (2007). Identification, activation, and selective in vivo ablation of mouse NK cells via NKp46. Proc. Natl. Acad. Sci. USA *104*, 3384–3389.

Warming, S., Costantino, N., Court, D.L., Jenkins, N.A., and Copeland, N.G. (2005). Simple and highly efficient BAC recombineering using galK selection. Nucleic Acids Res. *33*, e36.